

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 December 2001 (06.12.2001)

PCT

(10) International Publication Number
WO 01/92569 A2

(51) International Patent Classification⁷: C12Q 1/68

(21) International Application Number: PCT/GB01/02265

(22) International Filing Date: 23 May 2001 (23.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0013043.5 31 May 2000 (31.05.2000) GB
0013863.6 7 June 2000 (07.06.2000) GB

(71) Applicant (for all designated States except US): **TEPNEL MEDICAL LIMITED** [GB/GB]; Unit 8, St. George's Court, Hanover Business Park, Altrincham WA14 5UA (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **OULTRAM, John, Douglas** [GB/GB]; 15 Granby Road, Stretford, Manchester M32 8JL (GB). **MULROONEY, Conor, Joseph** [GB/GB]; 8 Colonial Road, Heaviley, Stockport, Cheshire SK2 6PJ (GB). **COUTTS, Jacqueline, Clare** [GB/GB]; 6 Buckingham Drive, Blenheim Gardens, Winsford, Cheshire CW7 1FA (GB).

(74) Agent: **ATKINSON, Peter, Birch; Marks & Clerk**, 83-85 Mosley Street, Manchester M2 3LG (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/92569 A2

(54) Title: FORMULATION FOR POLYMERASE CHAIN REACTION AND VESSEL CONTAINING SAME

(57) Abstract: A formulation for use in effecting a Polymerase Chain Reaction, a dried composition of reagents including reaction buffer, dNTPs, at least two primers and a polymerase. The formulation is re-hydratable to be capable of effecting amplification of a target nucleic acid sequence of interest. The formulation incorporates a fluorescent reporter molecule capable of reporting by homologous detection the presence of amplified nucleic acid produced by the Polymerase Chain Reaction.

FORMULATION FOR POLYMERASE CHAIN REACTION AND VESSEL
CONTAINING SAME

The present invention relates to formulations for use in effecting a Polymerase Chain Reaction and also to vessels containing such a formulation and which are intended for use in conducting such a reaction.

The Polymerase Chain Reaction (PCR) is one of the key tools in field of DNA diagnostics and enables the rapid and specific amplification of very small amounts of DNA sequences of interest. PCR is routinely performed either to obtain sufficient DNA for subsequent manipulation (e.g. for DNA sequencing or sub-cloning) or to identify the presence/absence of a specific nucleotide sequence in a large background of non-specific sequences.

Whilst PCR has revolutionised molecular biology, its ability to generate massive numbers of molecules of a particular DNA sequence from very few initial molecules can be a handicap in certain diagnostic situations. This is because of the ease of contaminating a PCR with the products of a previous reaction. Once contamination of a work area has arisen it can be very difficult to eradicate. This has lead to the adoption of complex and expensive methods of minimising this risk, such as purpose built laboratories, or laboratory areas, enzymatic pre- or post- PCR treatment of samples and controlling the use of such items as pipettes, lab coats etc, within defined work areas.

The PCR reaction itself generally consists of a number of preparative steps including the addition of a buffer solution, dNTP mix, primer solutions and usually a separate MgCl₂ solution followed by the addition of target and DNA polymerase. Many of the reagents can be included in a "master mix" that is then dispensed singly to individual reactions. Other reagents, usually the target and polymerase enzyme must be added individually to reaction tubes, which involves pipetting very low volumes (sub to

low µL) which can lead to considerable reaction variability. The complexity of the steps involved in optimising the performing PCR in this fashion requires a high degree of expertise in those entrusted with its performance and constant vigilance and monitoring of contamination issues.

A proposal to overcome the disadvantages set out in the previous paragraph is set out in US-A-5 861 251 (Park et al) which discloses a ready-to-use PCR reagent formulation obtained by lyophilisation of an aqueous mixture comprising a reaction a DNA polymerase, buffer, MgCl₂, dNTPs, PCR primers, glucose or glucitol as a stabilising and sedimenting agent, and a water soluble dye selected from bromphenol blue, xylene cyanol, bromocresol red and cresol red. This lyophilised mixture has the advantage that it simplifies the multi-step PCR manipulation in that all components (except target) for effecting amplification are included in the pre-prepared mixture such that all that is required is addition of an aqueous sample containing (or potentially containing) the target. Furthermore, the water soluble dye facilitates identification of complete mixing of the PCR reagent and test sample and saves the trouble of adding a sample loading buffer which is otherwise required for analysis of PCR products. As a result, the formulations of US-A-5 861 251 provide the advantage of avoiding carry-over contamination into the PCR reaction mix. However detection of the amplified product is effected by running the product mixture on a gel. This necessitates opening of the tube, to apply the product mixture to the gel, thus once again giving rise to the possibility of cross-contamination.

It is an object of the present invention to obviate or mitigate the above-mentioned disadvantages.

According to a first aspect of the present invention there is provided a formulation for use in effecting a Polymerase Chain Reaction, the formulation comprising a dried composition of reagents including reaction buffer, dNTPs, at least two primers and a

polymerase and said formulation being re-hydratable to be capable of effecting amplification of a target nucleic acid sequence of interest characterised in that the formulation incorporates a fluorescent reporter molecule capable of reporting by homologous detection the presence of amplified nucleic acid produced by the Polymerase Chain Reaction..

As with that proposed in US-A-5 861 251, the formulation of the invention is such that only a single addition of aqueous target sample to the formulation is required to produce an aqueous reaction mixture containing all necessary components for PCR amplification of target nucleic acid sequence.

The formulation of the invention does however have the significant additional advantage that the presence, in the formulation, of the fluorescent reporter molecule means homologous detection may be used. Thus the progress of the reaction may be followed by real-time detection techniques avoiding the need for post-reaction manipulation of the product mixture (e.g. transferring the mixture to a gel, or even opening a vessel in which the product mixture is contained) thereby avoiding any possibility of cross-contamination. This has dramatic consequences for the set-up of laboratories that PERform PCR-based diagnostic reactions as, currently, extreme care has to be taken during the performance of the reaction to prevent cross-contamination. Using the present invention, no particular contamination controls would be needed other than those routine in a molecular biology laboratory. There are also additional benefits, including having much more defined reaction conditions (as essentially all the reactants could come from the same batch, convenience, longer shelf life etc).

The invention also provides, according to a second aspect thereof, a vessel (e.g. a reaction tube) containing a pre-measured amount of the formulation of the invention.

The vessels (with premeasured formulation) may be provided with a suitable closure element and supplied to end users who, after removal of the closure element merely, need only to add the aqueous sample and then re-close the vessel. The end user may be a person in a laboratory where the PCR reaction is then effected. Alternatively the end user may be out "on-site" collecting samples which can then be added to the vessel as soon as collected, the vessel then being sent to a laboratory for conducting the PCR reaction.

Conveniently, after addition of the aqueous sample to the vessel the latter is closed with a non-removable closure element so that there can be no possible interference with the PCR reaction which, as described above, may be effected and monitored without the need to remove the closure element. For this purpose, the inner surface of the vessel (adjacent the mouth thereof) and the outer surface of the closure element may be provided with inter-engageable formations allowing insertion of the closure element into the vessel but preventing withdrawal therefrom. These inter-engagable formations should be positioned such that the closure element is capable of being removable provided that it has not been inserted into the vessel beyond a certain degree. As such, the dried composition may be incorporated into the vessel and the closure element removably applied thereto. Subsequently the closure element may be removed to permit addition of the sample and then subsequently inserted sufficiently far into the vessel so that it becomes non-removable.

The formulation of the invention may be prepared by lyophilisation of an aqueous solution of the required components, e.g. by lyophilisation using the procedures disclosed in US-A-5 861 251. Preferably the solution includes a stabiliser which may for example be glucose, glucitol or trehalose.

The dried formulation of the invention may be such that, per ml of reconstituted reaction medium, it comprises:

<u>Component</u>	<u>Amount</u>
Polymerase	0.01-0.04 units
Primers	0.1-10 pmoles
Fluorescent Reporter	0.1-10 pmoles
KCl	10-100 nmoles
Tris-HCl	10-20 nmoles
Triton X100	0.5-5mg
MgCl ₂	0.5-10 nmoles
DNTP(each)	50-500 pmoles
Stabiliser (e.g. trehalose)	0.1-15% w/w

Once the lyophilised sample has been rehydrated by addition of aqueous sample, the PCR reaction may be conducted by procedures well known in the art, e.g. using thermal cycling.

The fluorescent reporter molecule included in the formulation of the invention may for example be one which reports a change in the amount of double stranded DNA present in the reaction, e.g. an intercalating dye such as Ethidium Bromide, CyBr Green or PicoGreen. Alternatively the fluorescent reporter molecule may be one which works in conjunction with a quencher moiety so as to be capable of reporting on the presence of specific nucleotide sequences in the mixture and may, for example, be a TaqMan probe, Molecular Beacon, Sunrise primer and Scorpion primer (Registered Trade Marks).

The polymerase may be a DNA polymerase and may be a thermally stable polymerase, e.g. Taq polymerase. There is however a deficiency with Taq in that it becomes active (though at low efficiency) at relatively low temperatures. As a result, mis-primed reactions can occur at these low temperatures before the PCR begins. Thus,

re-hydration of the reaction mix, if not carried out at or above the annealing temperature of the primers, could lead to non-specific reactions occurring that will reduce the efficiency of the PCR and could lead to false positive reactions being reported. It is therefore preferred that the polymerase in the formulation of the invention is a "hot-start" polymerase. "Hot-start" polymerases are known in the art and are such that a heating step is required to activate the polymerase (which has typically been inactivated with an antibody). The "hot-start" polymerase should be one for which the "means" of inactivation of the enzyme (e.g. an antibody) must be able to withstand the drying/rehydration procedure. The advantage that the use of such an enzyme confers is that the re-hydration of the dried reagent composition can occur at ambient temperature without initiating potentially ruinous side reactions prior to heating the sample and cooling to annealing temperature at which only desired reactions can occur.

CLAIMS

1. A formulation for use in effecting a Polymerase Chain Reaction, the formulation comprising a dried composition of reagents including reaction buffer, dNTPs, at least two primers and a polymerase and said formulation being re-hydratable to be capable of effecting amplification of a target nucleic acid sequence of interest characterised in that the formulation incorporates a fluorescent reporter molecule capable of reporting by homologous detection the presence of amplified nucleic acid produced by the Polymerase Chain Reaction..
2. A formulation as claimed in claim 1 wherein the fluorescent reporter molecule is capable of reporting a change in the amount of double stranded DNA in the Polymerase Chain Reaction.
3. A formulation as claimed in claim 2 wherein the fluorescent reporter molecule is an intercalating dye.
4. A formulation as claimed in claim 3 wherein the intercalating dye is selected from Ethidium Bromide, CyBr Green and PicoGreen
5. A formulation as claimed in claim 1 wherein the fluorescent reporter molecule is one which works in conjunction with a quencher moiety so as to be capable of reporting the presence of specific nucleotide sequences in the mixture.
6. A formulation as claimed in claim 1 to 5 wherein the polymerase is a DNA polymerase.
7. A formulation as claimed in claim 6 wherein the polymerase is a thermally stable polymerase.

8. A formulation as claimed in claim 7 wherein the thermally stable polymerase is Taq.
9. A formulation as claimed in claim 6 or 7 wherein the polymerase is a "hot-start" polymerase.
10. A vessel for conducting a polymerase chain reaction therein, said vessel containing a pre-measured amount of the formulation as defined in any one of claims 1 to 9.
11. A vessel as claimed in claim 10 which is a reaction tube.
12. The combination of a vessel as claimed in any one of claims 10 and 11 and a closure element for closing the vessel after the addition of aqueous sample thereto.
13. The combination as claimed in claim 12 wherein the closure element is capable of effecting irreversible closure of the vessel.
14. The combination as claimed in claim 13 wherein the inner surface of the vessel (adjacent the mouth thereof) and the outer surface of the closure element are provided with inter-engageable formations allowing insertion of the closure element into the vessel but preventing withdrawal therefrom.
15. A method of effecting a polymerase chain reaction comprising adding an aqueous sample potentially containing target nucleic acid sequence to a vessel as claimed in claim 10 or 11 or the vessel of the combination as claimed in claim 12 or 13; effecting the polymerase chain reaction, and effecting fluorescence detection of the product mixture in situ in the vessel.

16. A method as claimed in claim 15 when used with the combination of claim 12 or 13, the method additionally comprising the step of inserting the closure element into the vessel after addition of the sample and prior to effecting the polymerase chain reaction.

17. A method as claimed in claim 16 when used with the combination of claim 14 wherein the step of inserting the closure element into the vessel effects irreversible closure.